Comparison of the structure and organization of the *rrna* operons of *Bouteloua gracilis* and *Zea mays*

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[1]Unidad de Biotecnología, Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales y Agropecuarias, Km 6.5 Carretera Celaya-San Miguel de Allende, Celaya, Gto. 38110, México; and [2]Laboratorio de Biotecnología 1, Facultad de Ciencias Químicas, Circuito Universitario s/n, Campus Universitario #2, C.P. 31125, Apartado Postal. 669, Chihuahua, Chih., México. *Received 26 April 2010, accepted 17 September 2010.*

Aguado-Santacruz, G. A., Betancourt-Guerra, D. A., Siquerios-Cendón, T., Arévalo-Gallegos, S., Rivera-Chavira, B. E., Nevarez-Moorillon, G. V., Moreno-Gómez, B. and Rascón-Cruz, Q. 2011. **Comparison of the structure and organization of the *rrna* operons of *Bouteloua gracilis* and *Zea mays***. Can. J. Plant Sci. **91**: 107–116. We studied the genomic structure of *Bouteloua gracilis* chloroplast DNA (cpDNA) and compared it with the sequenced ribosomal RNA spacer region from other cereals. This will allow us to understand chloroplast topology and the recombination ability of cpDNA. The development of potential tools for biotechnology applied to cereals can be focused through the study of cpDNA in family related grasses, such as *B. gracilis*. cpDNA was prepared from green *B. gracilis* and *Zea mays* plants using a modified NaCl method. A 2332 bp intergenic spacer (IGS) region (rrna*16S-trnI-trnA-rrna23S*) from *B. gracilis* was sequenced, which showed great similarity (at least 92%) to IGS region from *Z. mays*, *Oryza sativa* and *Saccharum officinarum*. A physical map constructed by Southern hybridization using *pet*A, *psb*A, *psb*D, *ndh*A, *rbc*L, *16S* and *23S* rDNA digoxigenin-labelled probes showed low organizational resemblance to maize cpDNA. Moreover, when compared to a similar fragment of *Z. mays*, a 239 bp intron deletion was found in the *trnI* gene in the *B. gracilis* cpDNA. Restriction and hybridization analyses suggested that the *B. gracilis* cpDNA has a molecular weight of 130 Kb. We expect that the findings reported in this work can be a baseline for increasing our knowledge in chloroplast organization in grasses and for the development of molecular tools.

Key words: Chloroplast DNA, cpDNA, *Bouteloua gracilis*, maize, physical map, restriction

Aguado-Santacruz, G. A., Betancourt-Guerra, D. A., Siquerios-Cendón, T., Arévalo-Gallegos, S., Rivera-Chavira, B. E., Nevarez-Moorillon, G. V., Moreno-Gómez, B. et Rascón-Cruz, Q. 2011. **Comparaison de la structure et de l'organisation des opérons *rrna* chez *Bouteloua gracilis* et *Zea mays***. Can. J. Plant Sci. **91**: 107–116. Les auteurs ont examiné la structure génomique de l'ADN des chloroplastes (cpADN) de *Bouteloua gracilis*, puis l'ont comparée à la région de l'espaceur de l'ARN ribosomique des autres céréales qu'on a séquencé. L'exercice permettra de comprendre la topologie des chloroplastes et la capacité de recombinaison du cpADN. Il est possible de faciliter l'élaboration d'outils de biotechnologie en vue d'une application aux céréales en étudiant le cpADN des graminées qui leur sont apparentées, comme *B. gracilis*. Le cpADN a été extrait de plants verts de *B. gracilis* et de *Zea mays* par la méthode au NaCl modifiée. Les auteurs ont séquencé un espaceur intergénique de 2 332 bp (IGS) (rrna*16S-trnI-trnA-rrna23S*) de *B. gracilis*, qui s'est avéré d'une grande similitude (au moins 92%) avec la région IGS de *Z. mays*, d'*Oryza sativa* et de *Saccharum officinarum*. L'hybridation de Southern a permis la construction d'une carte physique à partir des sondes de rADN *pet*A, *psb*A, *psb*D, *ndh*A, *rbc*L, *16S* et *23S* marquées à la digoxigénine. Celles-ci ont révélé une faible ressemblance organisationnelle avec le cpADN du maïs. Par ailleurs, les auteurs ont découvert un intron de suppression de 239 bp sur le gène *trnI* du cpADN de *B. gracilis* quand ils l'ont comparé à un fragment similaire de *Z. mays*. Les analyses de restriction et d'hybridation laissent croire que le cpADN de *B. gracilis* a un poids moléculaire de 130 Kb. On s'attend à ce que les résultats de ces travaux pavent la voie à une meilleure connaissance de l'organisation des chloroplastes chez les graminées et conduisent au développement d'outils moléculaires.

Mots clés: ADN des chloroplastes, cpADN, *Bouteloua gracilis*, maïs, carte physique, restriction

Chloroplasts are multifunctional intracellular organelles where central processes to plant functioning take place, including photosynthesis, starch synthesis, nitrogen metabolism, sulfate reduction, and fatty acid, DNA, RNA and ABA biosynthesis (Loveys 1977; Zeltz et al. 1993). They are also the target for compartmentalization of different important compounds involved in, for example, cellular division (Benková et al. 1999) and

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Abbreviations: IR, inverted repeat; **LSC**, large single copy region; **SSC**, small single copy region

the osmotic adjustment of plants (Gupta and Berkowitz 1999). The endosymbiotic theory of chloroplast refers to the theory that an independent organism (Cyanobacteria) was engulfed by a prokaryotic cell; chloroplasts have their own genome with a highly conserved organization (Raubeson and Jansen 2005) that is very distinct from the nucleus. Since the first complete plant chloroplast DNA (cpDNA) sequences were reported in tobacco (Shinozaki et al. 1986) and liverwort (Ohyama et al. 1986), an increasing amount of work has been completed to complement information about the structure and function of the chloroplast in higher plants. From the analysis of cpDNA we now know, for example, that most land plant chloroplast genomes contain a single circular chromosome composed of two copies of an inverted repeat (IR) that separate the large and small single copy regions (LSC and SSC). Although the overall structure of the chloroplast genome is conserved, variation in both gene content and sequence organization, including the loss of one copy of the inverted repeat, has been documented (Downie and Palmer 1992). Differences among genomes have also been attributed to the presence of inversions and additions/deletions found in noncoding DNA. While the conservative nature of chloroplast evolution has limited its use in phylogenetic analysis to the higher taxonomic levels, the intergenic noncoding regions may provide a source of increased sequence variation that could be useful in the study of relationships among more closely related species (Morton and Clegg 1993). In addition to deletions/additions, a second possible source of variation within noncoding DNA is the introns found within many chloroplast genes, for example the type II introns located in the tRNA genes *trn*A and *trn*I of the rRNA operon.

The family Poaceae is one of the largest families of flowering plants with approximately 10 000 species, including the world's most important crops. In recent years, a clear picture has formed of the evolutionary history of the grass family. Part of this information has been derived from restriction site maps of the chloroplast genome (Palmer et al. 1988; Warwick and Black 1991; Soreng and Davis 1998) and sequences of chloroplast genes, including *matK* (Hilu et al. 1999), *ndhF* (Clark et al. 1995), *rbcL* (Barker et al. 1995), *rpoC2* (Barker et al. 1999) and *rps4* (Nadot et al. 1994). Besides its importance on phylogenetic and taxonomical grounds, the analysis of the chloroplast sequence, structure and organization has been further impelled by the advantages of chloroplasts as transgene expression compartments for applications in biotechnology (Bock 2007) and due to their important participation in the response of plants to water stress (García-Valenzuela et al. 2005). Examples of applications in basic science include the study of plastid gene transcription, mRNA editing, photosynthesis and evolution. Examples of biotechnological applications include the incorporation of transgenes in the plastid genome for containment and high-level expression of recombinant proteins for pharmaceutical and industrial applications. Plastid transformation is routine only in tobacco (Bock 2007).

The tribe Cynodonteae, of subfamily Chloridoideae, includes several warm- and cool-season forage and turf grasses genera, some of which, such as *Astrebla*, *Bouteloua*, *Buchloë*, *Chloris*, *Cynodon* and *Hilaria*, are of great economic importance. Unlike the *Lolium* and *Festuca* grass species, for which biotechnological frameworks are well-established, less attention has been devoted to other important forage and turf grasses, such as blue grama grass.

Blue grama grass, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud., has recently emerged as a new model species for the functional genomics of warm season grasses because of its high tolerance to drought (Barnes et al. 1984), salinity (Weiler and Gould 1983), cold (Weaver and Zink 1946), fire (Dwyer and Pieper 1967) and grazing (Alward et al. 1999), as well as to toxic levels of Cadmium. *B. gracilis* is a C4, drought-tolerant, perennial grass, native to the North American grassland, which extends from southern Canada to central Mexico (Weaver and Clements 1938; Hitchcock 1950). In this region, blue grama can account for 75–90% of the net primary production of grasses (Coffin and Lauenroth 1992) and yields high-quality forage for domestic livestock and native fauna (Stubbendieck et al. 1986).

In the past few years, a considerable effort has been made by our research group to develop genetic and molecular tools for the study of *B. gracilis*, including the identification and isolation of mRNA and proteins related to osmotic stress response, genetic diversity characterization of native populations (Aguado-Santacruz et al. 2004) and the development of efficient and reproducible protocols for in vitro plant regeneration (Aguado-Santacruz et al. 2001) and genetic transformation (Aguado-Santacruz et al. 2002). Development of the first chlorophyllic cell system within the Poaceae (Aguado-Santacruz et al. 2001) has facilitated the generation of this knowledge. Using this model cell line, in this research, we are now reporting the first analysis of the structure and organization of the cpDNA of *B. gracilis* and its comparison to that of one of the most important cereals for human consumption, maize (*Z. mays*). Because the challenge in chloroplast transformation for flowering plants is in the design of ad hoc systems, we expect this information to support the development of efficient and reproducible chloroplast transformation systems and the elucidation of the participation of these organelles in the water stress response of grasses.

MATERIALS AND METHODS

Plant Materials

Maize seeds of inbreed line LPC13 were obtained from the Maize Breeding Program of INIFAP at Celaya,

Gto., Mex. LPC13 seeds were germinated in 2-L pots and the six-leaf stage seedlings were transferred to 20-L plastic containers where they were grown to maturity in a greenhouse. In contrast, *B. gracilis* plants, regenerated in vitro from the embryogenic, highly chlorophyllic cell line TADH-XO (García-Valenzuela et al. 2005), were maintained in 10-L plastic containers in the greenhouse. Additionally, we utilized this cell line as source material for DNA chloroplast isolation. This cell line was obtained from culturing shoot apices-derived green calli in liquid MPC medium, containing 1 mg L^{-1} 2, 4-D, 2 mg L^{-1} BAP and 40 mg L^{-1} adenine dissolved in Murashige and Skoog medium (1962), as described previously (Aguado-Santacruz et al. 2001). This cell line was subcultured every 20 d, transferring 1 mL of the cell suspension into 24 mL of fresh MPC medium.

For chloroplast DNA isolation from all of the plant sources previously mentioned, leaves of maize and *B. gracilis* were collected from adult plants and used fresh, while *B. gracilis* chlorophyllic cells were harvested at day 6 of growth, and then stored at −70°C for subsequent cpDNA extraction.

cpDNA Isolation and Restriction

Green leaf tissue (40 g) or 20 g of cultured chlorophyllic cells was used as the starting material for chloroplast DNA isolation. The method utilized in this work for the isolation of *B. gracilis* and maize cpDNA significantly reduced nuclear DNA carryover. The plant material was homogenized in a Waring blender (three to five 5-s bursts at high speed) with a 6:1 buffer:tissue ratio, using an ice-cold extraction buffer containing NaCl 1.2 mol L^{-1}, Tris-HCl 50 mmol L^{-1} pH 8.0, EDTA 5 mmol L^{-1}, 0.05% BSA and 0.1% 2-mercaptoethanol. After rupture, the material was filtered through four layers of cheesecloth and the recovered filtrate was then centrifuged at 1000 rpm for 15 min at 4°C. The resulting pellet was re-suspended in 200 mL of wash buffer (350 mmol L^{-1} Sorbitol, 50 mmol L^{-1} Tris, pH 8.0, 25 mmol L^{-1} EDTA) and the mixture centrifuged at 4500 rpm for 15 min at 4°C. This washing process was repeated again, but this time the pellet was dissolved in 1 mL of suspension buffer (100 mmol L^{-1} Tris-HCl pH 7.2, 50 mmol L^{-1} EDTA, 100 mmol L^{-1} NaCl, 0.2% 2-mercaptoethanol). Chloroplast lysis was achieved by adding SDS with a final concentration of 0.6% and incubation at 60°C for 5 min. Debris were separated by centrifugation and the chloroplast lysate was subsequently placed in a new tube and washed twice with phenol-chloroform and once with chloroform-isoamylic alcohol. cpDNA was recovered by precipitation by adding 1/10 vol 5 M sodium acetate and 2.5 vol absolute ethanol and with centrifugation at 12 000 g for 20 min. The resulting pellet was washed twice with 70% ethanol and suspended in sterile H_2O.

Once the cpDNA concentration was spectrophotometrically determined, it was digested with *Sal*I, *BamH*I, *Sac*I and *Pst*I alone or by combining two of these enzymes (Invitrogen, CA). The digested cpDNA was electrophoresed in 0.7% agarose gels, stained with ethidium bromide and then recorded under UV light.

Cloning and Sequencing of a 16S-23S Ribosomal Operon cpDNA Fragment

An UTR-containing fragment of 5 Kb between the 16S-23S ribosomal subunits of cpDNA was amplified by LA PCR (Cline et al. 1996). Amplifications were performed in a Palm Cycler thermocycler (Corbett Research, Australia) using 1 × PCR Buffer, 25 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} dNTP, 2 mmol L^{-1} forward and reverse primers and 2.5 u of Pfx pol (Invitrogen, CA). The long and accurate LA PCR was performed with 30 cycles that included a step of 30 s denaturation at 95°C, 40 s annealing at 58°C and a 5 min extension at 68°C. Primers were designed to amplify the conserved 5′ and 3′ regions of the *16S* subunit: *16S*1, 5′-CATGCTTAACACATGCAAGT-3′ and *16S*2, 5′-TACCGTACTCCAGCTTGGTAG-3′. Primers employed for the *23S* subunit amplification were *23S*1, 5′-CTAAGGTAGCGAAATTCCTTGT-3′ and *23S*2, 5′-CTTGGCTACCCAGCGTTTACC-3′.

Amplicons derived from these PCR reactions were blunt-ended and cloned into the *Eco*RV site of the pMOSBlue plasmid vector (Invitrogen, CA). The ligation mixture was transformed into Select96 *Escherichia coli* competent cells (Promega, Madison, WI), plated onto LB agar containing 100 g L^{-1} ampicillin and 80 g L^{-1} X-Gal at a density allowing the isolation of individual colonies and then incubated at 37°C overnight. The sequence of intergenic spacers between 16S rDNA-23S rDNA was obtained by multicolor fluorescent labeling technology in an ABI PRISM 310 (Applied Biosystems, CA) through successive overlapping events and using the specific reverse primers ITS1, 5′-CTGGCCAGGTTTGAAGTCAT-3′, ITS2, 5′-GACCCGAAGATGTGGATCAT-3′ and forward primers ITS3, 5′-TGCTGAGTTGGAATCCCATTA, ITS4, 5′-TTTGGGAATCTCCGGATCTA-3′. The nucleotide sequences obtained were compared with those deposited in public databases using the BlastX search algorithm. Detailed multiple sequence alignments were obtained with ClustalX2.0 (Thompson et al. 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007) and the BioEdit Sequence alignment editor (DNAmlk subroutine) (Hall 1999).

The sequence was annotated at the NCBI genome database. Localization of several chloroplast genes within the cloned fragments was carried out by hybridization to PCR-amplified genes: *psbA*, *ndhA*1, *rbcL*, *petA* and *psbD*. Probes designed to hybridize against the previously restricted cpDNA (*B. gracilis*, *Z. mays*) were

prepared by the incorporation of dUTP-Digoxigenin in PCR reactions using the following primers sets: *psbA* 1, 5′-CGAAAGTACAAGCCTGTGGG-3′; 2, 5′-GATGCAGCTTCCCAAATTGG-3′: *ndhA* 1, 5′-CCCCTGGGACTGCTTCAAGC-3′; 2, 5′-CTAATA GATTGAGCGGCAGC-3′: *rbcL* 1, 5′-GCGTTGGA GGGACCGTTTTG-3′; 2, 5′-TCCTGAGTGAAA AAGATACC-3′: *petA* 1, 5′-CCCATTTTTGCACAG CAGGGTTATG-3′; 2, 5′-CCCTCCGAAACAAGAA GTT-3′: *psbD* 1, 5′-TATGACTATAGCCCTTGGTA-3′; 2, 5′-TAGAACCTCCTCAGGGAATA-3′.

Southern Transfer and DNA Hybridization

To determine the size and localization of the above-mentioned genes within the *B. gracilis* chloroplast genome, digested cpDNAs were transferred to a nylon membrane using a 25 mmol L^{-1} sodium phosphate buffer, pH 6.5 (Southern 1975). Filters were prehybridized at 65°C in 5X SSC containing 0.01% lauroylsarcosine, 0.02% SDS, and 1% blocking reagent for 4 h. Filters were hybridized overnight at 65°C with the aforementioned buffer using the procedure described in Rascon-Cruz et al. (2003). The hybridization buffer contains 30 ng/ml of total cpDNA *Z. maize Bam*HI nick translated digoxigenin-labelled probes, and *pet*A, *psb*A, *psb*D, *ndh*A, *rbc*L, *16S* and *23S* rDNA genes using PCR digoxigenin-labelled probes.

RESULTS

Cloning and Sequencing of a 16S-23S Fragment of *B. gracilis*

A 5 Kb DNA fragment containing the DNA sequences located between the 16S and 23S genes of the *B. gracilis* cpDNA was amplified by LA PCR and subsequently ligated into the blunt pMOSBlue vector. Subsequently, a 2332 bp fragment of the intergenic spacers located between 16S and 23S rRNA was sequenced from both strands. The PCR product was cloned in a TA pCR-topo system and clones derived from DH5 transformation were analyzed by restriction. Sequencing was carried out using the appropriate sets of primers mentioned in the Materials and Methods section. The selected clone was sequenced and assembled by overlapping the PCR-generated fragment. The assembled sequence was further deposited in the GenBank under the accession number EU282003. The sequence fragment from *B. gracilis* was uploaded to the Dual Organellar GenoMe Annotator (DOGMA) database (Wyman et al. 2004), and results showed that it shares the gene cluster *16S rDNA-trnI-trnA-23S rRNA* with other monocots (maize and rice). A schematic representation of the rDNA operon of *B. gracilis* is depicted in Fig. 1a. Also, this sequence was compared with the non-redundant database of the NCBI to corroborate its identity. The sequenced cpDNA fragment of *B. gracilis* showed 96% similarity (836 to 2294 nucleotides) to the corresponding fragments found in the cpDNA inverted repeats of *Z. mays* (97662 to 99105 nucleotides), *Sorghum bicolor* (98541 to 100005 nucleotides) and S. *officinarum* (98374 to 96931 nucleotides), accounting for 1439 nucleotides from the total. On the other hand, the 16S-23S region in *B. gracilis* was 239 bp shorter than Z. *mays, S. bicolor and O. sativa.* A sequence alignment using NCBI homology results (pairwise alignment) as a homologous selection was done. Sequences recovered in pairwise alignment (NCBI) including gaps were used for multiple alignment analysis (Fig. 1b). Sequence comparison of the 16S-23S region (2332 nucleotides) with similar sequences of *S. officinarum*, *Z. mays*, *S. bicolor*, *O. sativa*, *Lolium perenne*, *Agrostis stononifera*, *Secale cereal*, *Triticum aestivum* and *Hordeum vulgare* showed that the blue grama operon was 230 bp shorter than that of Z. *mays*. A more detailed analysis of the IGS region showed a single large deletion located between the 895 and 1141 positions of the *trnI* intron (Fig. 1b).

The deletion found in the *trnI* intron of blue grama is absent in the maize, cane, sorghum and rice plastome. We found characteristically T-rich containing DNA block "hotspots" bordering the deleted regions. In our study, the blue grama deletion at the *trn*I was delimited by T-rich blocks, (TTCGAGCCTTTTTTT 9/16 dT; 12/16 pyrimidines at the 5′ endpoint) (Fig. 1b, continuous box), and a 16-bp block, TATTTATC TATCTCTTGACTCG (11/21 dT; 16/21 pyrimidines), at the 3′ endpoint (Fig. 1b, continuous box). Regions upstream and downstream from the blue grama intron deleted region exhibited high homology ($>95\%$). Also, a shorter deletion of the 147 nucleotides appears at positions 994 to 1141 of the *trn*I intron of the ryegrass, creeping bentgrass, rye, wheat and barley plastome, flanked by an additional T-rich block in all aligned sequences that correspond to the 5′ endpoint (Fig. 1b, dotted box).

Using this alignment data (gramineous species), a phylogenetic tree was constructed in ClustalX2 using the Neighbor Joining method and analyzed in TreeView software (Fig. 2). As expected, *B. gra*cilis, as a member of the subfamily Chloridoideae, showed a higher degree of constancy rate with species classified within the Panicoid subfamily (monophyletic group), which includes maize (*Z. mays*), sugarcane (*S. officinarum*) and sorghum (*S. bicolor*), than with species included in other monophyuletic groups within the subfamily Pooideae, which includes bentgrass (*Agrostis*), barley (*H. vulgare*), wheat (*T. aestivum*), rye (*S. cereale*) and ryegrass (*L. perenne*), or Bambusoideae, which includes rice (*O. sativa*). A timeline of evolution using the maximum likelihood method with a molecular clock was tested using this alignment data in Bioedit (DNAmlk subroutine) and analyzed in MEGA4. The highest likelihood tree (Ln-4556.02) was chosen from among competing topologies (Fig. 2b). The phylogenetic tree of monocots reveals that the highest level transition/transversion ratios in Panicoid clade are assigned to *B. gracilis,*

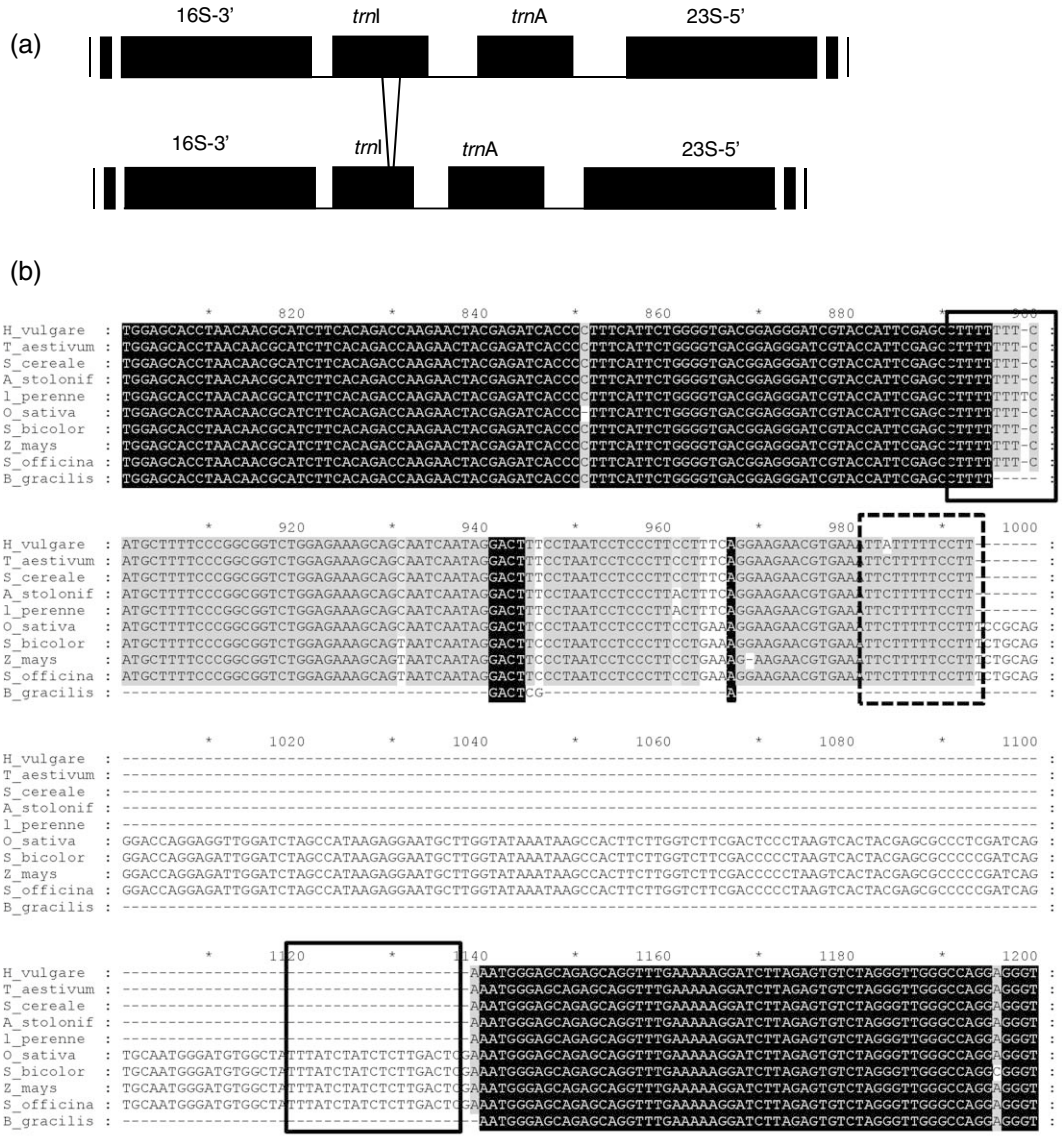

Fig. 1. Analysis of the amplified 5 Kb DNA fragment containing the DNA sequences located between the 16S and 23S genes of the *B. gracilis* cpDNA. (a) Schematic representation of the *16S rRNA-trnI-trnA-23S rRNA* operon of maize (upper) and blue grama (lower). (b) Sequence comparison of *Zea mays* (GenBank accession No. AY928077), *Hordeum vulgare* (GenBank accession No. E11541), *Nicotiana tabacum* (GenBank accession No. Z00044) and *Bouteloua gracilis* (GenBank accession No. EU282003, from this work). Gaps introduced during alignment are indicated by dashed lines (-), while black and gray filled spaces indicate sequence identity and partial identity, respectively. Boxes indicate deletion endpoints (dashed, 5′; continuous, 3′).

and to *S. cereal* in Pooideae clade. Thus, this grass (*B. gracilis)* is the less evolved organism among the monocots group.

Determination of the cpDNA Size of *B. gracilis* Using Endonuclease Analysis

Bouteloua gracilis and *Z. mays* cpDNA were analyzed by restriction enzyme cutting to examine the purity of the isolated cpDNA and to determine the size of the respective plastomes. A large number of fragments of varying size were generated by restriction enzymes *BamH*I, *Eco*RI, *Hind*III/*Eco*RI, *Hind*III and *Sal*I (Fig. 3). The electrophoretic patterns of bands indicated an apparent low similarity between the cpDNA genomes of blue grama and maize. The total size of the *B. gracilis* cpDNA fragments generated by the four enzymes was between 133.3 and 137.8 (mean = 135.5 Kb), while that of maize was between 138 and 145.1 (mean = 142 Kb).

Nick translated probes from total maize cpDNA were used to analyze differences between the *Bouteloua* and maize cpDNA′s. Major differences were clearly visible

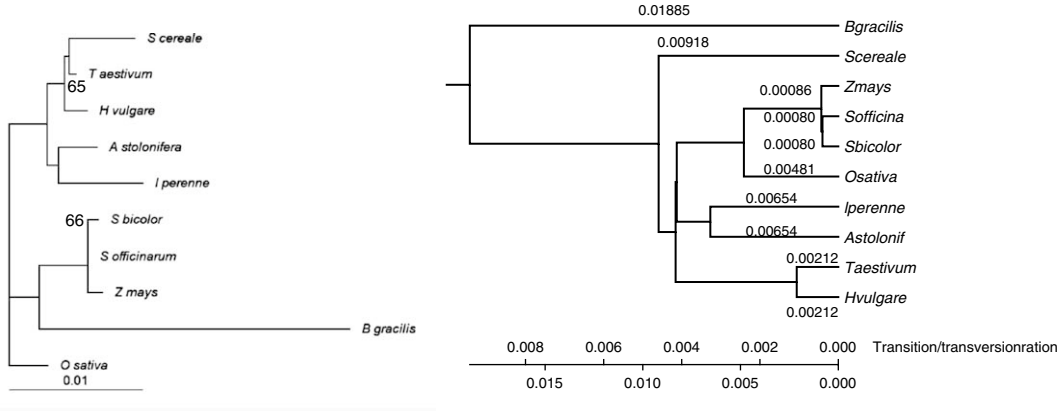

Fig. 2. Phylogenetic relationship in monocots of the sequenced fragment (EU282003) from *B. rrna* operon to the corresponding sequences of different grasses found at the NCBI database. (a) The tree was inferred by the neighbor-joining method based on the number of synonymous substitutions in chloroplasts genes. Most nodes in each phylogeny received 93–100% of the bootstrap replicates, and only values less than 90% are shown for each node. The horizontal branch lengths are proportional to the number of substitutions per site, and *Oryza sativa* was selected as an out-group. (b) Maximum-likelihood analysis.

when these probes were hybridized against the cpDNA of these two species restricted with enzymes *BstX*I, *EcoR*V, *Hpa*I, *Nco*I and *Sca*I (Fig. 4). A large number of fragments ranging from 20 to 0.5 Kb were observed. Clear differences in the restriction patterns were found with almost all of the enzymes tested, except *Hpa*I. Despite belonging to the same family, Poaceae, specific differences were found along the two sequenced *rrna* operons, particularly within the IGS regions (Fig. 1).

Physical Map of the *B. gracilis* Plastome

The order of the restriction fragments of the *B. gracilis* cpDNA was determined by hybridization to seven *Z. mays* cpDNA probes and from further confirmation by homology to the *Z. mays* chloroplast genome (NC_001666). For example, the hybridization pattern obtained in *B. gracilis* and maize cpDNA when using a *nadhA* probe was the same (4 Kb) and corresponded to a fragment located at the junction SSC-IR (Fig. 5). Based

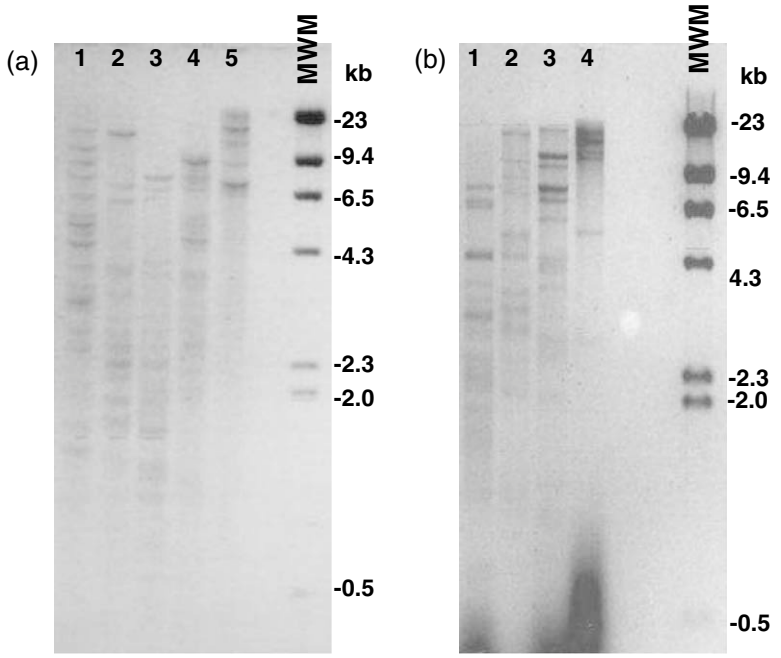

Fig. 3. Restriction fragment patterns. (a) *Bouteloua gracilis* cpDNA digested with: Lane 1, *BamH*I; lane 2, *EcoR*I; lane 3, *Hind*III/*EcoR*I; lane 4, *Hind*III; and lane 5, *Sal*I. (b) *Zea mays* cpDNA digested with the following: lane 1, *Hind*III/*EcoR*I; lane 2, *EcoR*I; lane 3, *Hind*III; and lane 4, *Sal*I. Fragments were electrophoresed for 18h in 0.8% agarose gels. MWM: Lambda/*Hind*III.

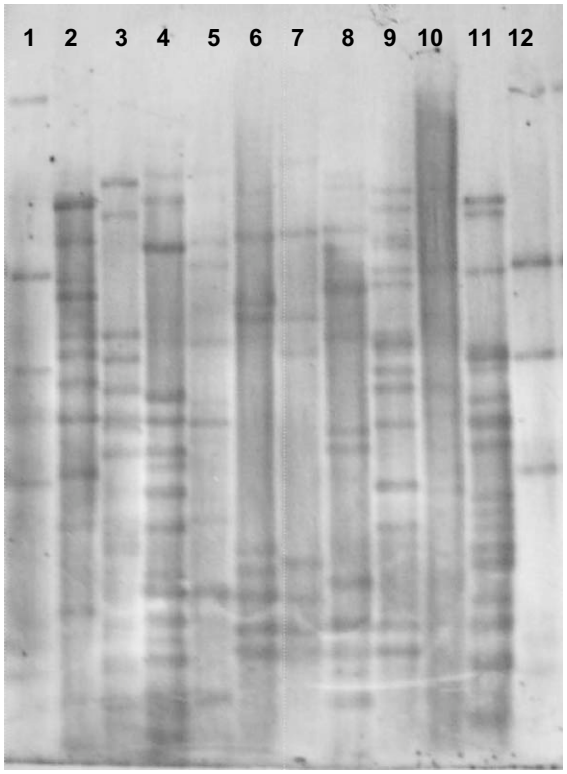

Fig. 4. Southern blot analysis of the *Bouteloua gracilis* (Bg) and *Zea mays* (Zm) cpDNA using a maize cpDNA nick translated digoxigenin-labeled probe. Lane 1, lambda/*Hind*III-digoxigenin labeled marker; lane 2, Bg/*BstX*I; lane 3, Zm/*BstX*I; lane 4, Bg/*Eco*RV; lane 5, Zm/*Eco*RV; lane 6, Bg/*Hpa*I; lane 7, Zm/*Hpa*I; lane 8, Bg/*Nco*I; lane 9, Zm/*Nco*I; lane 10, Bg/*Sca*I; lane 11, Zm/*Sca*I; and lane 12, lambda/*Hind*III-digoxigenin labeled marker.

on the patterns generated with the five restriction enzymes and their hybridization to the labeled probes, a complete map of the *B. gracilis* cpDNA was constructed (Fig. 5). The structural organization of the chloroplast genomes of *B. gracilis* and *Z. mays* was basically the same as that observed in other grasses, containing a large single-copy (LSC) region of approximately 82 Kb, a small single-copy (SSC) region of approximately 13 Kb, and two inverted repeats (IRs) of approximately 25 Kb. The localization of the seven chloroplast genes was assigned on the physical map (Fig. 5).

DISCUSSION

Cloning and Sequencing of a 16S-23S Fragment of *B. gracilis*

The chloroplast of *B. gracilis* showed the same operon organization (Fig. 1) found in land plants (Maier et al. 1995). The deleted intron segment in trnI explains the size difference during homology alignment. In the chloroplast genomes of higher plants, the genes encoding *trn*I and *trn*A contain group II introns and are part of the *16S rRNA–trnI–trnA–23S rRNA–4.5S rRNA–5S rRNA* operon (Shinozaki et al. 1986). Group II introns comprise the majority of noncoding DNA in most chloroplast genomes (Jurica and Stoddard 1999) and are among the fastest evolving regions known in the plastome (Watson 2000). Chloroplast group II introns are increasingly providing a rich source of sequence characters for intrageneric and intrafamilial phylogeny estimation (Kelchner 2002). Johnson and Hattori (1996) proposed the existence of preferred sequences (''hotspots'') for deletion formation at the *trn*I and *trn*A introns, which seems to be generated by a conserved mechanism(s). However, the IR region of the chloroplast genome is known to have a lower rate of change than the single copy region (Jansen and Palmer 1987; Johansson and Jansen 1993). Noncoding regions of cpDNA diverge through insertion/deletion changes that are sometimes site-dependent (Yang and Wang 2007) and intermolecular recombination between distinct tRNA genes is possible (Hiratsuka et al. 1989; Ogihara et al. 2002).

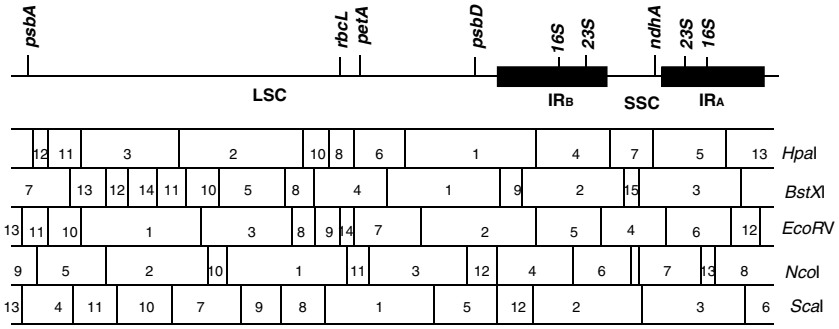

Fig. 5. Physical map of the *Bouteloua gracilis* cpDNA in a linear form showing the cutting sites for the restriction enzymes *Hpa*I, *BstX*I, *Eco*RV, *Nco*I and *Sca*I. IR_A and IR_B are the inverted repeat regions. SSC are the small single-copy and LSC the large single-copy regions. *psbA*, photosystem II D1; *rbcL*, ribulose bisphosphate carboxylase large chain; *petB*, cytochrome F; *psbD*, photosystem II D2; *16S*, 16S ribosomal RNA; *23S,* 23S ribosomal RNA; *ndhA*, NADH dehydrogenase ND1.

Phylogenetic Analysis

The phylogenetic tree obtained from the comparison of the *Bouteloua rrna* operon against the non-redundant database of NCBI using the neighbor-joining method from Blast reflected, at the plastome level, the phylogenetic relationships previously shown by Kellogg (2001) for the gramineous species analyzed in this work (Fig. 2). Maximum-likelihood analysis with a molecular clock using only the IR region showed a high transition/transversion ratio (0.0087) when most of the monocots ranged around 0.004 (Fig. 2b). Extensive indels have been found in different members of Poaceae (Asano et al. 2004) and even within varieties of a particular species (Kanno et al. 1993). Micro structural changes, such as insertions, deletions and inversions in the chloroplast genome of higher plants, can be extremely useful both for resolving phylogenetic relationships among basal lineages of angiosperms (Graham et al. 2000; Ingvarsson et al. 2003) and for inferring relationships among more closely related taxa (Golenberg et al. 1993; Kelchner and Clark 1997; Kelchner 2000). Introns and intergenic spacers from the chloroplast genome are now increasingly used also for phylogenetic comparisons (Liu et al. 2006). Recent examples employing this strategy for the analysis of chloroplast DNA evolution include studies of the intergenic regions between rbcL and ORF136 in wheat species (*Triticum* and *Aegilops*) (Ogihara et al. 1988), within almost the same region (rbcL-psaI) in seven species of the grass family (Poaceae) (Morton and Clegg 1993), between trnL and trnF in the Crassulaceae (van Ham et al. 1994), and between psbA and trnH in alfalfa (*Medicago sativa*) and petunia (*Petunia hybrida*) (Aldrich et al. 1988). These studies were directed towards the potential use of deletions/additions as phylogenetic markers. However, analysis of the sequences surrounding a small number of deletions/additions has led several authors to suggest potential mechanisms for deletion formation. The comparison of a small set of representative coding or intergenic sequences derived from a large number of species has been used to perform phylogenetic studies and it is well known that differences among cpDNA are mainly found within IGS (Soltis et al. 2004).

Determination of the cpDNA Size of *B. gracilis* Using Endonuclease Analysis

It was possible to obtain an enriched fraction of cpDNA from *B. gracilis* with the combination of two purification protocols: tissue lysis and chloroplast isolation in high salt isolation buffer (Bookjans et al. 1984) and sorbitol containing buffer for wash and resuspension (Koldner and Tewari 1975). Restriction analysis of *B. gracilis* and *Z. mays* showed that our size values are in concordance with those reported for the cpDNA of other grasses (135–140 Kb) (Fig. 4) (Yaneshita et al. 1997). This result supports the idea of developing tailor-made chloroplast transformation systems due to differences in number and gene organization in the chloroplast genome (Maliga 2002), even within the highly conserved and synthetic plastomes of the grass family (Devos and Gale 2000).

Physical Map of the *B. gracilis* Plastome

The locations of the IR and SSC junctions have been known to vary among different cpDNAs (Kim and Lee 2004; Chang et al. 2006). As determined by Southern blot hybridization in this study, the gene arrangement of the maize and blue grama cpDNAs was similar to that found in other grasses. However, sequence differences were found, mainly within the NTS regions. Introns are under less selection pressure than exons, and consequently, intron sequences have a higher rate of gain and loss than exons (Lin et al. 2006).

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